Genotypic analysis of *Candida albicans* isolates obtained from removable prosthesis wearers

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Abstract

Aims: To assess of the genotypic diversity of *Candida albicans* isolated from removable prosthesis wearers, with and without denture-related stomatitis (DRS). The occurrence of different genotypes in pathological and control cases was investigated.

Methods and Results: One hundred and sixty-four isolates of *C. albicans* obtained from different oral cavity locations were compared by randomly amplified polymorphic DNA (RAPD). The coherence of this analysis was confirmed by genotyping a selected group of isolates with pulsed field gel electrophoresis (PFGE). Among the 164 isolates, 150 were grouped into seven groups on the basis of their RAPD patterns. Three of these groups (comprising 54 isolates) had significant ($\alpha < 0{\cdot}10$) predominance of clinical or control cases. For the other isolates, no significant differences were observed between control and DRS cases. Occasionally, more than one genotype was found in the same person. These findings were sustained by PFGE analysis. No relevant associations between the genotypic patterns and pathology level were found.

Conclusions: This study evidenced that *C. albicans* with similar genotypes may be found in individuals with DRS and in control cases.

Significance and Impact of the Study: This conclusion hints the involvement of other aetiological factors that alone or in association with *C. albicans* may trigger the emergence of DRS.

Keywords
Candida albicans, denture-related stomatitis, genotyping, oral cavity, randomly amplified polymorphic DNA.

Introduction

Denture-related stomatitis (DRS) is an inflammatory process that mainly involves the palatal mucosa when it is covered by complete or partial dentures (Baena-Monroy *et al.* 2005). DRS is frequent among denture wearers, with prevalence values ranging from 24% to 60% (Barbeau *et al.* 2003; Webb *et al.* 2005). It is estimated that almost half of the Portuguese population wearing upper removable prosthesis exhibits this pathology (Figueiral *et al.* 2007). The condition may be classified in three types according to the clinical appearance of the inflamed mucosa, as proposed by Newton (1962). Type I refers to the initial stage of localized pin-point hyperaemia, type II is described as being a diffuse erythema and oedema, limited to the denture-bearing areas of the palatal mucosa, and type III develops when a hyperplastic reaction occurs resulting in a nodular lesion of the palate, often with associated atrophic areas, known as papillary hyperplasia.

Although the aetiology appears to be multiparametric, it is generally assumed that most of the cases of DRS are yeast related (Barbeau *et al.* 2003; Baena-Monroy *et al.* 2005). The mucosal surfaces are primary reservoirs of yeasts that, consequently, can also be found on the dental plaque and on the denture prosthesis (Webb *et al.* 1998; Eggimann *et al.* 2003). Therefore, it is usually assumed that the disease arises from the endogenous commensal strains (Eggimann *et al.* 2003). *Candida albicans* is amongst the most frequently isolated species from the oral mucosa (60–80%) (Richardson and Warnock 2003)

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and has been specifically related with the DRS (Bartie *et al.* 2001; Barbeau *et al.* 2003).

In the present study, *C. albicans* isolates from individuals wearing removable prosthesis, with and without symptoms of denture-related stomatitis, were genotyped by randomly amplified polymorphic DNA (RAPD) in order to assess the diversity of these yeasts in pathology and control cases. Strains isolated from different locations of the oral cavity were compared and possible associations between genotype and pathology were assessed.

Material and methods

Sampling and culture conditions

Patients from the Faculty of Dentistry of Porto University (FMDUP) Removable Prosthodontics Department, Portugal, were the population studied, with the consent of the local research ethics committee. All participants signed a declaration of informed assent according to the rules of the Declaration of Helsinki guaranteeing the necessary confidentiality of collected information. Inclusion criteria included: adults (over 18 years), of both sexes, with maxillary acrylic prosthesis having at least half palatal coverage, but who had not had a new or modified prosthesis within the previous 6 months. All patients under antifungal treatment were excluded. From the 140 participants analysed, *C. albicans* was isolated from 84, 57 with DRS diagnosed [according to Newton's (1962) classification] corresponding to a total of 118 isolates and 27 with no evidence of DRS, corresponding to 46 isolates. Among the isolates recovered from DRS cases, 38 were associated with type I, 47 with type II and 33 with type III. Of the 57 DRS cases diagnosed, 43 (75·4%) were detected in females and 14 (24·6%) in males. From the 27 controls, 15 (55·6%) were females and 12 (44·4%) were males. The participant ages ranged from 18 to 86 years with a median of 58 years in the DRS group and 67 in the control group.

Microbiological samples were obtained from (i) the palatal mucosa (32 isolates), (ii) the prosthesis fitting surface (64 isolates), (iii) the stimulated saliva (61 isolates) and (iv) the mouth rinses (seven isolates). Samples were plated onto Sabouraud dextrose agar (SDA) with gentamicin, chloramphenicol and TTC (SANOFI-Pasteur; Bio-Rad, Marnes-la-Coquette, France) and incubated for 48 h at 37°C. The resulting growth was quantified by counting the number of colony-forming units ml^{-1} and purified for identification. Yeasts were identified on the basis of morphological examination (production of germ tubes after incubation in human serum for 120 min at 37°C and production of chlamydospores when incubated under a sterile cover slip for 72 h at 25°C on corn meal agar containing Tween 80) and biochemical tests (carbon source assimilation, with ID32C identification kits; API System, Biomerieux, Marcy l'Etoile, France). These data are reported in Figueiral *et al.* (2007). Other phenotypic tests included the production of extracellular phospholipase (Price *et al.* 1982) and proteinase (Borst and Fluit 2003). The pure cultures were preserved at −80°C in broth medium supplemented with 15% (w/v) of glycerol.

Random amplified polymorphic DNA analysis

Chromosomal DNA was extracted from fresh SDA cultures using the GFX™ Genomic Blood DNA purification kit (Amersham Pharmacia Biotech, Freiburg, Germany) according to the manufacturer's recommendations. The RAPD reaction was performed using Ready-To-Go RAPD Analysis Beads (Amersham Pharmacia Biotech) using the primer 2 (5′-d[GTTTCGCTCC]-3′) provided by the kit. PCR was performed in a total volume of 25 μl containing 1 μl template DNA (∼50 ng of yeast chromosomal DNA), 5 μl of primer (0·8 mmol l^{-1}) and 19 μl water mixed with the Ready-To-Go RAPD Analysis Bead containing PCR buffer (30 mmol l^{-1} KCl, 10 mmol l^{-1} Tris–HCl, pH 8·3, 2·5 μg BSA, 3 mmol l^{-1} $MgCl_2$, 0·4 mmol l^{-1} dNTPs, 1 unit Amplitaq DNA polymerase). Thermocycling was performed in a BioRad ICycler with initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 1 min, annealing at 36°C for 1 min and extension at 72°C for 2 min with a final extension step at 72°C for 8 min. The amplification products were analysed by gel electrophoresis on a horizontal 2% agarose gel for 4 h at 125 V at room temperature in TBE 0·5X running buffer. A reference *C. albicans* strain, ATCC 10231, was included to each batch of samples as standard. Bands were visualized after ethidium bromide staining on a UV-light transilluminator and photographed with the Kodak DC120 Zoom Digital Camera.

Pulsed field gel electrophoresis analysis

The pulsed field gel electrophoresis (PFGE) analysis was conducted according to a previously described procedure (Chu *et al.* 1993; Lupetti *et al.* 1995), which included an endonuclease digestion of genomic DNA using *Sfi*I. The electrophoresis was conducted by the contour-clamped homogeneous electric field technique. Conditions comprised pulse times ramped from 5 to 90 s for 24 h at constant 200 V, with an angle of 106° at 14°C. The resulting gels were stained with ethidium bromide (0·5 μg ml^{-1} in distilled water) for 30 min. DNA bands were visualized under UV light and photographed as before.

Data analysis

The genotypic patterns were analysed and compared visually. Each band was scored as positive or negative for all isolates, and for each isolate, the presence or absence of each band was registered. The resulting matrix was interpreted using a hierarchical clustering method, based on Dice coefficient (SPSS 14·0 for Windows; SPSS Inc., Chicago, IL), where isolates were grouped according to their pattern resemblance. Dendrograms based on a pairwise distance ($1 - S_{AB}$) matrix of the DNA samples were generated by unweighted pair group method with arithmetic averages. The chi-squared test was used to analyse the relationship between control and pathological isolates, with a significance level of $P < 0{\cdot}10$.

Results

The RAPD profiles presented a maximum number of 15 well-defined bands ranging from 1000 to 4000 bp. These patterns allowed the clustering of 150 isolates in seven clusters differing in eight bands. Fourteen isolates were kept unclustered (Fig. 1).

In order to clarify the RAPD analysis, a PFGE study was performed with a total of 21 isolates, one from cluster 1, one from cluster 2, seven from cluster 3, seven from cluster 4, one from cluster 6, two from cluster 7 and two unclustered isolates. This analysis showed that isolates belonging to a same RAPD cluster presented the same PFGE pattern or differed by one or two bands. All the isolates unclustered or belonging to different clusters by RAPD analysis were observed to differ by three or more bands. These findings confirmed the coherency of the RAPD analysis and the genotypic diversity was further studied.

For the majority of the isolates, it was not possible to establish an association between sample origin, DRS or control cases and genotype. Nevertheless, 54 isolates integrated genotypic clusters that comprised predominantly DRS cases (C1 and C6) or controls (C5) (Table 1). These observations indicate that similar *C. albicans* genotypes may be distributed among healthy and diseased individuals. Similar genotypes of *C. albicans* were observed in different sampling sites within the oral cavity, with exception observed in 35% of DRS patients and 18·5% of healthy individuals (data not shown).

Table 1 Distribution of *Candida albicans* randomly amplified polymorphic DNA genotypes from denture-related stomatitis and control cases

Cluster (*n*)	Healthy isolates, *n* = 38	DRS isolates, *n* = 112	χ^2	α
C1 (21)	2/21 (9·5%)	19/21 (90·5%)	2·775	0·096
C2 (23)	6/23 (26·1%)	17/23 (73·9%)	0·007	0·934
C3 (17)	3/17 (17·6%)	14/17 (82·4%)	0·531	0·466
C4 (22)	4/22 (18·2%)	18/22 (81·8%)	0·595	0·441
C5 (17)	16/17 (94·1%)	1/17 (5·9%)	42·522	0·000
C6 (16)	1/16 (6·3%)	15/16 (93·8%)	3·080	0·079
C7 (34)	6/34 (17·6%)	28/34 (82·4%)	1·062	0·303

Concerning the proteinase production, 82% of isolates were consistent producers and about 84% of these were DRS related.

Extracellular phospholipase production was observed in about half of the isolates (46%) and 79% of these were DRS related (Table 2). However, no correlation was obtained between the different production classes and RAPD clusters (data not shown).

Discussion

Denture-related stomatitis, the commonest form of oral candidosis, is present in almost 50% of Portuguese denture wearers. Although the aetiology appears to be multiparametric, it is generally assumed that the presence of the opportunist pathogen *C. albicans* in oral cavity is considered an important factor in the development of this inflammation.

The RAPD-based typing was used to assess the diversity of *C. albicans* isolates, as it has been described as a simple, rapid and reliable discriminatory method for clinical epidemiological studies of *Candida* infections (Pujol *et al.*

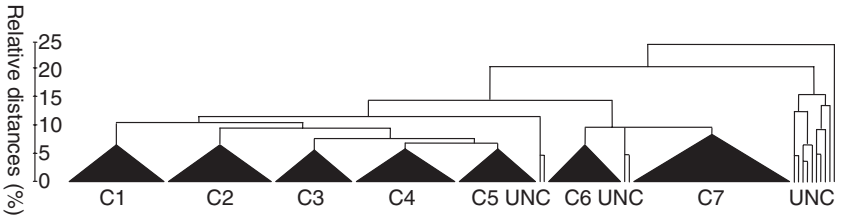

Figure 1 The dendrogram was constructed from randomly amplified polymorphic DNA data generated by primer set 2 using a hierarchical clustering method, based on Dice coefficient, which clustered 150 isolates into seven groups, defined with relative rescaled distances below 15%, and 14 isolates were kept unclustered. C, cluster; UNC, unclustered isolates.

Table 2 Distribution of the proteinase and phospholipase production among the randomly amplified polymorphic DNA clusters (C), unclustered isolates (UNC) and isolates from denture-related stomatitis (DRS) and control cases

Cluster (*n*)	Proteinase production, *n* = 135	Phospholipase production, *n* = 76
C1 (21)	16/21 (76·2%)	11/21 (52·4%)
C2 (23)	18/23 (78·3%)	8/23 (34·8%)
C3 (17)	15/17 (88·2%)	13/17 (76·5%)
C4 (22)	20/22 (90·0%)	9/22 (40·9%)
C5 (17)	14/17 (82·4%)	2/17 (11·8%)
C6 (16)	13/16 (81·3%)	8/16 (50·0%)
C7 (34)	28/34 (82·4%)	18/34 (52·9%)
UNC (14)	11/14 (78·6%)	7/14 (50·0%)
Control cases (46)	36/46 (78·3%)	16/46 (34·8%)
DRS cases (118)	99/118 (83·9%)	60/118 (50·8%)
Total	135/164 (82·3%)	76/164 (46·3%)

1997; Jain *et al.* 2001; Hossain *et al.* 2003; Boriollo *et al.* 2005). For example, Jain *et al.* (2001) recommended this technique to differentiate *C. albicans* strains resistant to fluconazole from its susceptible counterparts isolated from AIDS patients. In spite of this and considering the reproducibility pitfalls of the RAPD method, it was considered adequate to compare the reliability of the RAPD-based clustering with that obtained with PFGE, referred as a more robust method (López-Ribot *et al.* 2000; Goering 2004; Boriollo *et al.* 2005). This genotyping method supported the RAPD-based profiling and hence a deeper analysis and discussion of results were made.

Every RAPD cluster presented isolates from both DRS and control cases in proportions similar to those of the starting population. The only exceptions were clusters C1, C5 and C6, which, nevertheless, included DRS and control cases' isolates. Altogether, these results are congruent with those found by Song *et al.* (2005) in a similar study of RAPD-based characterization of oral yeasts. Also, the production of extracellular enzymes was observed to be independent of the genotype, as no association was observed between genotype and enzyme production (Table 2). These results are in the same line of evidence as those obtained by Hamal *et al.* (2004) in an RAPD-based study of clinical *Candida* proteinase producer strains.

A similar genotype was observed to be disseminated by several individuals, a fact that may be explained on the basis of an exogenous transmission, namely, a person-to-person transmission as reported by Fanello *et al.* (2001). In contrast, strains with different genotypes were found in the same oral cavity, possibly resulting of genetic variations such as gene conversion and/or chromosomal translations (Song *et al.* 2005).

These results led us to the conclusion that, despite the high prevalence of *C. albicans* in DRS patients (Barbeau *et al.* 2003), this organism may not be responsible for the emergence of the pathology. In fact, in previous studies, *C. albicans* was not isolated from about 20% of the patients with DRS diagnosed (Figueiral *et al.* 2007). Taking together these evidences, it is possible to hypothesize about the involvement of other opportunists present in the oral cavity. Besides the virulence of *C. albicans* in DRS, also the host susceptibility (immunity system impairment, underlying diseases) and other exogenous factors such other micro-organisms, which importance should be studied in more depth.

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